Gene Flow

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We have demonstrated that the addition and expression of pesticidal genes in the broad host range entomopathogens *Metarhizium anisopliae* and *M. robertsii*, and the acridid specialist *M. acridum* can greatly improve pathogen performance against mosquitoes, grasshoppers, caterpillars, beetles, bed bugs etc. In particular, expression of different arthropod neurotoxins can reduce effective spore doses by 40-fold and survival times by 50%. More rapid killing of insects should provide better control, and lowering the effective dose means that equivalent control could also be achieved using less product. This means a cheaper as well as more effective product. We have also produced *Metarhizium* strains that efficiently express functional single-chain antibody fragments which is particularly significant as recombinant antibodies provide a vast array of potential anti-insect effectors, that could target, for example, hormone receptors. These would allow construction of very effective, highly specific, biopesticides with minimal negative environmental impact.

In the long run, molecular biological techniques will make it possible to engineer recombinant microbes that show narrow specificity for target pests and that persist in the environment, providing sustainable cheap control for much longer periods than existing chemicals. However, from the point of view of risk assessment, the issue of strain mutability and stability will be crucial to these endeavors, and we do not currently seek to permanently establish an engineered microbial agent in the environment. A science based regulatory system currently has three requirements for enhanced biocontrol agents: limited off site dispersal, poor long term persistence, and limited possibility of recombination between pathogens (Gressel, 2007 in Novel Biotechnologies for Biocontrol Agent Enhancement and Management. M. Vurro and J. Gressel (eds). 353-362). There is a possibility that transgenes could move horizontally from a biocontrol agent to another species, and there is evidence that points to horizontal transfer of genes in evolutionary times e.g., a cold shock protein in *M. anisopliae* that is very similar to sequences in *Pseudomonas* spp (Fang and St. Leger, 2010, Environ. Microbiol. 12: 810-820). The risk that this can happen in human time is exceedingly low, and the Stakeholders 2003 report put emphasis on developing genetically based containment that would reduce rather than eliminate risk.

Vertical gene transfer between sexually or asexually compatible organisms is a bigger threat. Most *Metarhizium* species have no known sexual stage. Discovering whether *M. anisopliae. M. robertsii* and *M. acridum* undergo sexual reproduction has important implications for understanding the evolution of new strains of these pathogens. A sexual stage would be very useful for strain improvement but a risk of introgression of transgenes to native populations in the field might then need to be mitigated by disruption of genes for sexual development. Recently completed genomes have shown that *Metarhizium* species have a complement of apparently functional genes whose orthologs in *N. crassa* and *A. nidulans* are known to be required for meiosis and sexual development (Gao et al., 2010, PLOS Genetics, In Press). Unlike *M. robertsii*, *M. acridum* has a strong RIP bias, and RIP is only functional when meiosis is frequent. More studies are required to understand the

importance of the RIP mechanisms in the evolution of *Metarhizium* genomes and to determine the frequency of meiosis.

The well known parasexual cycle that occurs in some fungi including *Metarhizium* provides another mechanism for hybridization. As with sexual hybridization there are numerous barriers between vegetative fusion of different fungal species with the major one being vegetative incompatability**,** which results from heterokaryon incompatability proteins (HIP) that block exchange of DNA. *M. acridum* has fewer (25 genes) HIPs than *M. anisopliae* (35 genes), which suggests that *M. acridum* may be less reproductively isolated than *M. anisopliae*. However, it is likely that *M. acridum* with its more specialized lifestyle and narrow environmental range encounters fewer genetically distinct individuals than the more opportunistic *M. anisopliae*. There is only one record in the literature of a gene passing between different fungal species. Sometime before 1941 the wheat pathogen *Pyrenophora triticirepentis* acquired the toxA virulence gene from another wheat pathogen in the same family. These two species can form anastomosis tubes (Friesen et al., 2006. Nat. Genet. 38, 953-956.).

We are exploiting easily identified marker genes (red and green fluorescent proteins), functional genomic tools for identifying genetic changes, and strains disrupted in genes that are implicated in soil survival and pathogenicity, to provide detailed knowledge of movement, persistence and modes of genetic change in transgenic strains of *M. robertsii* strain 2575. An overview and rational for our work is given in St. Leger (2008. J. Invertebre. Pathol. 98: 271-276). To determine if deployment of transgenic strains in field conditions was likely to result in gene transfer to other strains, we used allozyme analysis of native *Metarhizium* isolates at the field site to identify 13 distinct genotypes. One of the 5 commonest genotypes is closely related to 2575. None of the 13 genotypes were capable of hybridizing with 2575 in the laboratory by co-infection of insects. We also monitored strain stability and possible dissemination of genetic material by determining whether 2575 transformed with both *gfp* and *rfp* retained both marker elements in their original form. After screening 1000's of colonies rescued from the field we found none that had lost GFP or RFP indicating an absence of recombination events with strains lacking these markers. The introduced *M. anisopliae* is not dormant, as fluorescent *Metarhizium* can be isolated from insects, and about 1% of colonies isolated from soil samples in plots co-inoculated with RFP-ΔMcl1 and GFP-ΔMad2 contained both markers. Some of these were unstable diploids that spontaneously breakdown to form aneuploids.

Given possible regulatory requirements, we are developing mitigating technologies in which natural biological barriers intrinsic to the pathogen will be complemented with failsafe devices utilizing site specific recombinases and mitigating genes to prevent recombination of transgenes into native strains, as well as reduce persistence and dispersal of transgenics in field conditions. The risk of transgene introgression would seem very small with *M. robertsii* strain 2575. Even though insects are hot spots for recombination for at least some *Metarhizium* strains (Leal-Bertioli *et al.*, 2000, Mycol. Res. 104: 409-414), strain 2575 has barriers that prevent recombination *in insecta* with the very closely related strain 23 (identical strains according to allozyme analysis). In this case a minimum of anti-introgressional failsafe mechanisms could lower the risk to an infinitesimal level. The resulting products would have combined improved biocontrol potential with a level of containment greater than that of the inefficient non-transgenic biocontrol agent.